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# PROVISIONAL APPLICATION COVER SHEET

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**TITLE OF THE INVENTION** (280 characters max)

PREVENTION OF NORLEUCINE INCORPORATION INTO PROTEIN

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PATENT

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# PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

for

# PREVENTION OF NORLEUCINE INCORPORATION INTO PROTEIN

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## FIELD OF THE INVENTION

[0001] The invention relates to the preparation of heterologous proteins from microorganisms and, more specifically, to preventing or substantially eliminating the incorporation of norleucine into these recombinant heterologous proteins. The present invention provides the compositions and methods necessary to prevent the incorporation.

## BACKGROUND OF THE INVENTION

[0002] Norleucine is an analog of the amino acid methionine that can be misincorporated into a protein in the place of methionine. In *Escherichia coli* (*E. coli*) norleucine can be biosynthesized by the enzymes of the leucine biosynthetic pathway. When expressed in *E. coli* many heterologous proteins have norleucine mistakenly incorporated in places methionine residues should appear. The misincorporation of norleucine is undesirable because it usually results in the production of an altered protein, having less than optimal characteristics.

[0003] The amino acid norleucine (2-aminocaproic acid; 2-aminohexanoic acid; see Figure 1), first known to science from synthetic preparations made in 1870, attracted great interest after being claimed in 1882 by the chemist Ludwig Thudichum to have been found as one of the natural amino acids of proteins. Other workers seemed to confirm this finding, claiming in 1912–1913 to have found norleucine in proteins. These observations were ostensibly confirmed and extended by yet more laboratories during the following two decades. This body of literature was reviewed by Schmidt (1933), and led him to recommend that norleucine be added to the list of accepted constituent amino acids of proteins. However, within 12 years, it was conclusively shown that the analytical techniques employed by the earlier workers had misled them, and that norleucine did not naturally occur in proteins (Consden et al., 1945). The history of norleucine up to 1945, and the error in identifying it as a standard protein amino acid, is recounted in detail by Vickery (1972).

[0004] Prior to 1945, while norleucine was still considered to be a standard protein amino acid, nutritional studies with rats demonstrated that rather than being an essential amino

acid, norleucine was actually toxic (Rose, 1938). Norleucine was also shown to be toxic to *E. coli* and other species of bacteria. It was further observed that the growth inhibition of *E. coli* by norleucine was reversed by the addition of methionine to the growth medium, thereby establishing that norleucine is an analog of methionine (Harris and Kohn, 1941; Rowley, 1953; Adelberg, 1958; Rowbury, 1965; Karlstrom, 1965).

[0005] A review of these and other early reports that norleucine is inhibitory to a variety of species of bacteria is provided by Dittmer (1950). Moreover, Dittmer (1950) noted that norleucine is a structural analog of methionine by virtue of the fact that when the sulfur atom in methionine is replaced by a methylene group norleucine is the result (see Figure 1). Thus, norleucine was recognized to be an amino acid antagonist, and a structural analog, of methionine. Norleucine attracted significantly more interest than most amino acid analogs, since it was so well characterized and readily available—aspects stemming from the time when norleucine was thought to be a standard protein amino acid.

[0006] The first report of the incorporation of exogenously supplied norleucine into protein was that of Rabinowitz *et al.* in 1954, who observed that exogenous norleucine was incorporated into protein in rat Ehrlich ascites carcinoma cells. A similar observation was made a year later when it was shown that exogenous norleucine could be incorporated into casein in cows (Black and Kleiber, 1955).

[0007] These findings were followed, in 1956, by a demonstration that exogenous norleucine was also incorporated into protein by *E. coli* (Munier and Cohen, 1956). This observation was confirmed by later work (Nisman and Hirsch, 1958), and the phenomenon was also shown to occur in *Staphylococcus aureus* (Anfinson and Corley, 1969).

[0008] Shortly thereafter, it was shown that the incorporation of exogenous norleucine into *E. coli* protein occurred at the positions where methionine residues normally occurred in the proteins (Cohen and Munier, 1959; Munier and Cohen, 1959; Cowie et al., 1959). This discovery was also confirmed by later work (Neale and Tristam, 1963; Pine, 1967; Kerwar and Weissbach, 1970; Zipori, 1976). The early research into the use of norleucine as an analog of methionine, and its incorporation into protein (when supplied exogenously to a variety of organisms) in place of methionine, was reviewed by Cohen and Gros (1963) and by Meister (1965).

[0009] By the mid-1960's it was widely known that exogenously supplied amino acid analogs that are incorporated into protein can have their incorporation blocked by the corresponding natural amino acid, especially when the natural amino acid is present in excess. The literature of that time provides several references establishing this general rule; including those found in Richmond (1962) and Fowden et al. (1967). Within a few years it was appreciated that for an amino acid analog to be incorporated into protein, it must compete with the naturally utilized amino acid for charging onto the corresponding tRNA (Pine, 1978, and Horton and Boime, 1983). These general rules for the incorporation of amino acid analogs into protein were highlighted by specific examples, including that the methionine analog norleucine was blocked from being incorporated into protein by the presence of methionine (Fowden et al., 1967; Pine, 1978; and Barker and Bruton, 1979).

[0010] Several studies independently demonstrated that the *E. coli* methionine-tRNA could be charged with norleucine *in vitro* and that this aberrant charging was inhibited by methionine (Trupin et al., 1966; Bruton and Hartley, 1968; Lemoine et al., 1968; Old and Jones, 1975; Old and Jones, 1977). Moreover, Old and Jones (1976) found that norleucine inhibited formation of methionyl-tRNA in an *E. coli* in vitro system; specifically, they showed that the level of methionine charging onto methionine-tRNA decreased gradually with increasing levels of norleucine.

[0011] *In vivo* studies also demonstrated that increased methionine pools reduced the incorporation of norleucine into protein. Fowden et al. (1967), in a review on amino acid analogs and their effects on *E. coli* and other organisms, stated (at page 91): "A general characteristic of all toxic analogs, whether synthetic or of natural origin, is that their toxic effects are specifically reversed by the normal protein amino acid which is antagonized by the analog.", and (at page 92): "an analog, prior to incorporation into protein, must be activated and transferred to a specific transfer-RNA. The analog therefore must compete with the structurally related protein amino acid at the surface of an aminoacyl-tRNA synthetase". Fowler (at page 136), referring to the 1964 Ph.D. thesis of S. Neale (University of London), further stated that "the amount of norleucine incorporated into alkaline phosphatase of *E. coli* K-12 under derepressed conditions was greatly reduced and the abnormally eluting enzyme was not apparent. Incorporation of the analog into the

purified enzyme and into gross cell protein was decreased due to increased supplies of intracellular methionine".

[0012] Others have also demonstrated *in vivo* that low methionine levels typically produce relatively high norleucine incorporation. The level of norleucine incorporated into protein was increased in experiments employing mutants of *E. coli* unable to make their own methionine, especially when the methionine in the growth medium was exhausted (Yariv and Zipori, 1972; Naider et al., 1972; Brown, 1973). This same observation was made with *Staphylococcus aureus* (Anfinson and Corley, 1969). Brown (1973) used a mutant of *E. coli* unable to make its own methionine, grown in a medium containing a high ratio of norleucine to methionine, to prepare proteins with norleucine at the amino-terminus and at internal residues. Barker and Bruton (1979) studied norleucine incorporation into protein in *E. coli*, reporting in detail on the effects of different ratios of norleucine to methionine on the charging of methionine tRNA with norleucine, and to the subsequent incorporation of norleucine into protein. They demonstrated that the incorporation of norleucine into protein was dependent on the intracellular ratio of norleucine to methionine; significant incorporation of norleucine into protein occurred at a high ratio, and greatly reduced incorporation of norleucine into protein occurred at a low ratio.

[0013] It was clear to these workers, as discussed above, that norleucine was not a standard protein amino acid. Indeed, they concluded that norleucine did not even occur in nature as a free amino acid. However, this conclusion was disproved by the observation that *Serratia marcescens*, an organism closely related to *E. coli*, is able to biosynthesize norleucine when the leucine biosynthetic system is derepressed (Kisumi et al., 1976, 1977). In this organism, the enzymes of leucine biosynthesis were shown to be responsible for the biosynthesis of the endogenous norleucine. The leucine biosynthetic enzymes have broad substrate specificities (Bogosian et al., 1989), and are capable of forming both leucine and the structurally related norleucine (see Figure 1). These reports by Kisumi et al. (1976, 1977) represent the first observations of norleucine as a naturally occurring substance.

[0014] Thus, by the late 1970's, a great deal was understood about norleucine structure, use, and synthesis. It was clear that norleucine was a structural analog of methionine that could be incorporated into protein by mis-charged methionine-tRNA. Furthermore, it was

clear that a sufficient amount of available methionine inhibited the incorporation of norleucine into protein by out-competing norleucine for the charging of methionine-tRNA. Finally, it was known that norleucine was a naturally occurring amino acid, synthesized in bacteria by the enzymes of the leucine biosynthetic pathway.

[0015] The stage was thus set for a series of observations made by Bogosian and co-workers in 1985 and published a few years later (Bogosian *et al.*, 1989). They found that norleucine was undesirably incorporated into both native and heterologous proteins being expressed in recombinant strains of *E. coli*. The level of norleucine incorporation into these proteins ranged from 5% to 15% of the normal methionine content. In this case the norleucine was not being supplied exogenously, but was being naturally synthesized in the *E. coli* cells. They showed that, in *E. coli*, the enzymes of the leucine biosynthethic pathway also biosynthesized norleucine, and that the norleucine so formed could be incorporated into protein in place of methionine.

[0016] In an effort to produce heterologous proteins with a reduced norleucine content, Bogosian *et al.* went on to show that the incorporation of norleucine into protein could be reduced by adding additional methionine to the culture medium. They also showed that norleucine biosynthesis could be reduced by supplying exogenous leucine to the culture medium (thereby repressing the induction of leucine biosynthetic enzymes). It was also shown that inactivating one or more of the genes of the *leu* operon, which encodes the leucine biosynthetic enzymes, prevented the biosynthesis of norleucine (however, a bacterial strain unable to make its own leucine requires the addition of leucine to the culture medium).

[0017] Bogosian *et al.* also demonstrated that the initial substrate for norleucine biosynthesis was 2-ketobutryate, an intermediate in the biosynthesis of isoleucine. Thus, another approach employed by these workers to prevent the biosynthesis of norleucine was to inactivate the *ilvA* gene. The *ilvA* gene encodes threonine deaminase, the enzyme that initiates isoleucine biosynthesis by converting threonine to 2-ketobutyrate. However, the *ilvA* mutant was also incapable of making its own isoleucine. Consequently, this approach necessitated the addition of isoleucine to the culture medium. Thus, while a variety of approaches were devised by these workers to reduce the incorporation of norleucine into

protein, they all required the addition of other amino acids (namely, methionine, leucine, or isoleucine) to the culture medium.

[0018] Other workers have made similar observations with other heterologous proteins expressed in recombinant *E. coli* strains. Norleucine was found to be incorporated into human interleukin-2 (Tsai et al. 1988, and Lu et al., 1988), recombinant human insulin-like growth factor I (Forsberg et al., 1990), human macrophage colony stimulating factor (Randhawa, 1994), human leptin (Liu et al., 1997), and human brain-derived neurotrophic factor (Sunasara et al., 1999). With these proteins, norleucine incorporation ranged from 5% to 20% of the normal methionine content.

[0019] Since norleucine is not a standard protein amino acid, it is desirable to minimize its incorporation into proteins in order to produce products that are as "natural" as possible (*i.e.* contain only the amino acids encoded by the DNA sequence). Previously devised methods for reducing the incorporation of norleucine into protein (Tsai et al. 1988, Bogosian et al., 1989, and Randhawa, 1994) were based on the prior art describing the biosynthesis of norleucine and the incorporation of norleucine into protein. That is, the prior art indicated that the biosynthesis of norleucine could be reduced by supplementation of the culture medium with leucine, thereby repressing the enzymes of leucine (and norleucine) biosynthesis. The art also indicated that inactivating the *ilvA* gene and/or one or more of the genes of the *leu* operon (namely *leuA*, *leuB*, *leuC*, and *leuD*) would reduce the biosynthesis of norleucine. Finally, the art indicated that supplementation of the culture medium with methionine would reduce the incorporation of norleucine into protein.

[0020] Thus, there are at least two approaches for preventing or reducing the incorporation of norleucine into heterologous proteins described in the existing art discussed above. (1) Inactivation of one or more of the genes encoding the biosynthetic enzymes necessary to produce norleucine. In *E. coli*, these genes include *ilvA*, *leuA*, *leuB*, *leuC*, and *leuD*. (2) Interference with the incorporation of norleucine into protein by supplementing the bacterial growth medium with methionine (or ALIMET® feed supplement, available from Novus International, Inc, St. Louis, Missouri, which *E. coli* can convert into methionine). That is, to competitively block norleucine incorporation into protein using this method, additional methionine accumulates inside the bacteria and

competes with the available norleucine for attachment to the methionine tRNA, thereby reducing norleucine incorporation into protein.

[0021] Inactivation of one or more of the genes *leuA*, *leuB*, *leuC*, or *leuD* as a means of reducing norleucine incorporation into protein was also described by Fenton *et al.*, in U.S. Patent No. 5,599,690. Supplementation of the culture medium with methionine as a means of reducing norleucine incorporation into protein was also described by Fenton *et al.* in the '690 patent, and by Brunner *et al.*, in U.S. Pat. No. 5,698,418. Brunner *et al.*, in the '418 patent, also provide a description of a means for reducing norleucine incorporation into protein by supplementing the growth medium with other amino acids, specifically, leucine or cysteine. All of these approaches have the disadvantage of requiring the supplementation of the culture medium with one or more amino acids.

[0022] Another approach for preventing norleucine incorporation (also described by Brunner *et al.* in the '418 patent) is to mutate the protein-encoding gene at the codons originally encoding methionine so that they encode other amino acids. This approach has the disadvantage of altering the primary (and perhaps secondary and tertiary) structure of the protein, which may result in significant and undesirable changes in the biological properties, activity, and usefulness of the protein.

[0023] As discussed above, all approaches described, in the existing art, as being effective for reducing the incorporation of norleucine into protein, require either the supplementation of the culture medium with one or more amino acids or the mutation of the gene encoding the protein's amino acid sequence to eliminate methionine codons. It is desirable in the biotechnology industry to be able to cultivate recombinant organisms in a simple chemically defined minimal medium, without the need to add any expensive supplements, such as amino acids while simultaneously reducing the incorporation of norleucine into proteins. Furthermore, it is also desirable to do so without altering the protein's primary amino acid sequence.

[0024] Prior to the discovery of the invention disclosed in the instant application, there was no method known in the art that was able to achieve the objective of reducing the incorporation of norleucine into protein without requiring the supplementation of the

culture medium with one or more amino acids and/or eliminating the methionine codons from the gene encoding the protein (thereby changing the protein's amino acid sequence).

[0025] Thus, there exists a need for a method of preventing or substantially reducing norleucine incorporation into heterologous proteins that does not require the use of expensive media amino acid supplements or alteration of the protein's amino acid sequence, but instead results in the proper incorporation of methionine into the protein.

## PROBLEM SOLVED BY THE INVENTION

[0026] The instant invention meets this need for an efficient and inexpensive means of preventing the incorporation of norleucine into heterologously expressed proteins. The instant invention meets this need by providing the methods and compositions necessary to prevent or substantially inhibit norleucine incorporation into heterologous proteins, without the necessity of supplementing the growth medium with amino acids or altering the protein's coding sequence to eliminate methionine codons.

[0027] The present invention meets this need by providing a method of reducing the incorporation of norleucine into protein by degrading the norleucine that the cell biosynthesizes. An important aspect of this invention is that it provides a means for achieving a reduction in the incorporation of norleucine into protein without necessitating the supplementation of the culture medium with any amino acids.

## SUMMARY OF THE INVENTION

[0028] While there is extensive prior art on the degradation of amino acids (for example by a broad substrate enzyme such as a general amino acid oxidase), there is no suggestion in the existing art to using such an approach for reducing endogenous levels of norleucine. Furthermore, using a broad substrate enzyme, such as an amino acid oxidase, would likely lead to the degradation of essential amino acids along with the norleucine and would, therefore, require that the growth medium be supplemented with these essential amino acids in order to replace the degraded amino acids. Nevertheless, there is no suggestion in the prior art describing an approach for reducing endogenous levels of

norleucine by degradation; much less suggesting how to do so without concomitantly reducing the levels of the required amino acids. In contrast, the instant invention provides for methods of preventing norleucine misincorporation into protein without having to supplement the growth medium with any amino acids.

[0029] Living organisms degrade excess amino acids to metabolic intermediates that can be used for other purposes. The major pathway of amino acid degradation starts with an oxidative deamination reaction that removes the alpha-amino group from the amino acid (Stryer, 1995). While little is known concerning the degradation of norleucine, the few studies that have been conducted indicate that oxidative deamination is also the first step in the breakdown of norleucine. Oxidative deamination of norleucine would yield 2-ketocaproic acid (2-ketohexanoic acid; see Figure 1) and ammonia. Bender and Krebs (1950) observed oxidation of norleucine by amino acid oxidases of cobra venom and *Neurospora crassa.* Kinnory et al. (1955) reported that in rat liver homogenates norleucine degradation was by transamination and decarboxylation reactions, which yielded 2-ketocaproic acid, valeric acid, and beta-hydroxyvaleric acid. Greenberg (1961) reviewed this work and proposed a pathway by which norleucine was degraded first to 2-ketocaproic acid, which in turn was degraded to valeric acid and carbon dioxide, then to beta-ketovaleric acid, then to propionic acid and acetic acid.

[0030] The studies that have been published on the degradation of norleucine by bacteria suggest that this is an ability possessed by very few species of bacteria. Indeed, the degradation of norleucine by *Clostridium difficile* and *Peptostreptococcus anaerobius*, to the exclusion of other related species, is used as the basis of rapid identification tests for these pathogens (Nunez-Montiel et al., 1983; Turgeon et al., 1990).

[0031] While few studies have been published on the ability of bacteria to degrade norleucine *in vivo*, it is known from *in vitro* studies of several bacterial amino acid degradative enzymes that, in addition to their normal role in degrading standard protein amino acids, some of these enzymes also exhibit a low level ability to degrade norleucine.

[0032] For example, *in vitro* studies of phenylalanine dehydrogenase from *Thermoactinmyces intermedius* indicated that both the wild-type enzyme and a variant

designated CS2 (with the substrate-binding domain of leucine dehydrogenase) were capable of degrading norleucine (via oxidative deamination) with 6% and 70%, respectively, of the activity against phenylalanine (Kataoka et al., 1993).

[0033] Also, Turnbull et al. (1997) reported, based on *in vitro* studies, that wild-type leucine dehydrogenase and valine dehydrogenase from various species of bacteria (*Streptomyces*, *Thermoactinomyces*, *Clostridium*, *Bacillus*, and *Corynebacterium*) were capable of degrading norleucine via oxidative deamination. Other enzymes that might degrade norleucine include other amino acid dehydrogenases, such as alanine dehydrogenase and glycine dehydrogenase, aminotransferases (also known as transaminases), amino acid dehydratases, and various amino acid oxidases.

[0034] Glutamate dehydrogenase (GDH) is an enzyme that degrades the amino acid glutamate via oxidative deamination to form 2-ketoglutarate and ammonia (see Figure 1). GDH from the organism *Clostridium symbiosum* has been crystallized and studied extensively.

[0035] A variant form of the *Clostridium symbiosum* GDH has been identified, in which the lysine residue at position 89 has been changed to a leucine residue (this is referred to as the K89L form of GDH). This GDH variant exhibits an increased ability to degrade norleucine (Stillman et al., 1999; Wang et al., 2001; Goyal et al., 2001). These references suggest that the K89L enzyme exhibited a high degradation rate for methionine and thus would not be useful for increasing the incorporation of methionine into proteins.

[0036] In contrast, as disclosed herein, the present invention provides for glutamate dehydrogenase (GDH) from *E. coli* (both wild-type GDH and variants comprising a lysine 92 to leucine, K92L, variation, of *E. coli* GDH) that efficiently degrades norleucine. That is, the instant invention provides for recombinant DNA molecules encoding the GDH proteins described as well as the recombinant proteins encoded. The instant invention also provides for methods for preparing recombinant strains of bacteria (*e.g.*, *E. coli*) with enhanced expression of the wild-type GDH gene and/or enhanced expression of the K92L variant form of *E. coli* GDH. In any embodiment of the instant invention, the modified cell has enhanced expression of the norleucine degrading enzyme as compared with its expression in the unmodified cell. Various embodiments of the

instant invention provide new protein expression systems in which heterologous proteins can be produced, where these proteins have a reduced norleucine content, and yet the bacteria are grown on a minimal medium; and, thus, do not require supplementation with any amino acids whatsoever (nevertheless, supplemental amino acids may be added). Also provided are the bacterial strains so produced.

[0037] The instant invention also provides for various means for reducing norleucine incorporation into heterologous proteins without the use of expensive amino acid supplements. That is, the methods of the instant invention require neither provision of exogenous amino acids (such as leucine, valine, or isoleucine) to compensate for the inhibition of a amino acid biosynthetic pathway, nor excessive methionine in order to competitively inhibit the incorporation of norleucine.

[0038] Notwithstanding that the instantly claimed invention effectively reduces norleucine incorporation into native or heterologous protein without the addition of amino acids supplements, various aspects of the instant invention also provide for the use of amino acid supplements in combination with cells having enhanced expression of one or more norleucine degrading proteins. By this means it is possible to even further reduce the incorporation of norleucine into proteins.

[0039] The instant invention provides methods and compositions that prevent or substantially eliminate the incorporation of norleucine into heterologous proteins by engineering a cell so that it degrades most or all of the norleucine that it synthesizes.

[0040] According to various embodiments of the invention, the norleucine degradation is accomplished by coexpressing a heterologous protein in the cell with enhanced expression of a protein, or enzymatically functional portion of a protein, that degrades norleucine.

[0041] As indicated above, other embodiments of the invention provide for recombinant DNA molecules capable of encoding a norleucine degrading protein and recombinant proteins capable of degrading norleucine.

[0042] Other embodiments of the instant invention provide for methods of purifying heterologous proteins having a reduced norleucine content.

## DESCRIPTION OF THE FIGURE

[0043] The following figure forms part of the present specification and is included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to this figure in combination with the detailed description of specific embodiments presented herein.

[0044] **Figure 1:** Shows the basic structures for the indicated amino acids. Also shown is the result of oxidative deamination of norleucine and glutamate.

## DESCRIPTION OF THE SEQUENCE LISTINGS

[0045] The following sequence listings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these sequences in combination with the detailed description of specific embodiments presented herein.

| SEQ ID NO: | Description |
|---|---|
| 1 | DNA sequence of wild-type *E. coli* glutamate dehydrogenase |
| 2 | Protein sequence of the wild-type *E. coli* glutamate dehydrogenase. |
| 3 | DNA sequence encoding the *E. coli* K92L glutamate dehydrogenase variant. |
| 4 | Protein sequence of the *E. coli* K92L glutamate dehydrogenase variant. |

## DEFINITIONS

[0046] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0047] As used herein, the term "heterologous protein(s)" preferably refers to a protein that is not expressed in the organism in an untransformed state. Put another way, it means that the protein is not native to the organism. In preferred embodiments of the

instant invention the term heterologous protein does not refer to any protein that is typically used as a "marker" (meaning a selection marker). Such "markers" include, but are not limited to antibiotic resistance genes and proteins capable of processing substrate so as to provide a colored product for a colorimetric assay.

[0048] As used herein the terms "coexpress", "coexpresses", and "coexpressed" refer to proteins/DNA molecules which are expressed in a cell as a result of a recombinant event. That is, at least one of the following is true: either the DNA and/or protein is expressed from an extra genomic vector (such as a plasmid) that has been introduced into the cell via a molecular biological technique; and/or the DNA/protein is expressed from a location in the cell's genome other than where the DNA sequence naturally occurs.

[0049] As used herein the term "enhanced expression" refers to the modification of a cell so that the expression of a particular RNA transcript or protein is increased in that modified cell as compared with the level of expression of that same RNA or protein in an unmodified cell. Means for enhanced expression contemplated as being part of the instant invention include, but are not limited to: expression of the gene from an extra-genomic DNA molecule (*e.g.* a plasmid); expression of the gene from a non-native location in the cellular genome; and/or expression of the gene from its native genomic location, but with modification of the gene's normal regulatory control system so as to stimulate expression or reduce suppression (that is any modification which increases the gene's expression).

[0050] Thus, as used herein the term "enhanced expression" refers to at least two distinct phenomena. One aspect of enhanced expression is the increased expression of a gene sequence already present in the cell (*e.g.* the increased expression, in *E. coli*, of RNA and/or protein that is native to *E. coli*, such as *E. coli* glutamate dehydrogenase) so that the RNA and/or protein encoded by the native sequence is present at higher levels than in the non-modified cell. A second aspect of enhanced expression is the expression of a "new" sequence that is not native to the cell. For example, the expression of a K92L glutamate dehydrogenase variant in an *E. coli* strain that did not previously produce the RNA or protein for the K92L variant of glutamate dehydrogenase. Thus, in sum

"enhanced expression" refers to both the "increased" expression of a native RNA/protein and the "new" expression of a non-native RNA/protein.

## DETAILED DESCRIPTION OF THE INVENTION

[0051] The instant invention provides for means useful to prevent or substantially eliminate the incorporation of norleucine into heterologous proteins. Various embodiments of the instant invention provide for methods that prevent incorporation of norleucine into proteins that are heterologously expressed. In certain aspects of this embodiment of the invention the incorporation of norleucine into heterologous proteins is prevented or substantially eliminated by coexpression of the heterologous protein in a cell with enhanced expression of a protein (or a catalytically active fraction thereof) that catalyzes the degradation of norleucine.

[0052] In one aspect of this embodiment the norleucine degrading protein is a glutamate dehydrogenase (GDH). In a particularly preferred aspect of this embodiment the norleucine degrading enzyme is GDH from *Escherichia coli* (*E. coli*). In another preferred aspect of this embodiment the norleucine degrading protein comprises a lysine 92 to leucine (K92L) variant of *E. coli* GDH. In a particularly preferred embodiment of the invention the heterologous protein is co-expressed in *E. coli* with enhanced expression of either an native *E. coli* GDH (or a enzymatically active fragment thereof) or a norleucine degrading protein comprising a K92L variant of *E. coli* GDH (or an enzymatically active fragment thereof). In any aspect of the current invention it is contemplated that the modified cell has enhanced expression of the norleucine degrading protein as compared with the protein's expression in the non-modified cell. In other aspects of the present invention the K92L variant may further comprise other variations from the native sequence. All such variants are considered to be part of the instant invention so long as they do not diminish the protein's ability to degrade norleucine to a degree where it is no longer useful according to the instant invention.

[0053] In other aspects of this embodiment of the invention the norleucine degrading protein may be selected from any protein found to produce a suitable degree of norleucine degradation. Thus, in addition to glutamate dehydrogenase, other proteins provided for use according to the instant invention include, but are not limited to

phenylalanine dehydrogenase (examples of such a phenylalanine dehydrogenase are wild-type and variant enzymes isolated from *Thermoactinmyces intermedius*, but this is not an exclusive set), leucine dehydrogenase, and valine dehydrogenase (exemplary leucine and valine dehydrogenases include, but are not limited to those obtained from *Streptomyces*, *Thermoactinomyces*, *Clostridium*, *Bacillus*, and *Corynebacterium*) other amino acid dehydrogenases, such as alanine dehydrogenase and glycine dehydrogenase, aminotransferases (also known as transaminases), amino acid dehydratases, and various amino acid oxidases.

[0054] In one particularly preferred embodiment of the instant invention the norleucine degrading protein is encoded by a DNA molecule comprising a sequence as provided in SEQ ID NO:1 or SEQ ID NO:3. In another preferred aspect of this embodiment of the invention the norleucine degrading protein has a peptide sequence comprising the sequence of SEQ ID NO:2 or 4.

[0055] In various embodiments of the invention the heterologous protein is any protein or protein fragment of interest that can be advantageously expressed in bacteria. In certain preferred aspects of this embodiment of the invention the heterologous protein is a somatotropin. In more preferred aspects of this embodiment the somatotropin is a human, bovine, equine, porcine, ovine, canine, or feline somatotropin. In a particularly preferred aspect of this embodiment the heterologous protein is bovine somatotropin (bST).

[0056] Other heterologous proteins to which the instant invention is drawn include, but are not limited to human interleukin-2, recombinant human insulin-like growth factor, human growth factor, human macrophage colony stimulating factor (M-CSF), human leptin, and human brain-derived neurotrophic factor. These proteins are listed as being exemplary only, the list is not exclusive. Accordingly, any heterologous protein for which the exclusion of norleucine is desired or necessary may advantageously be produced in accordance with the instantly described invention.

[0057] Other embodiments of the instant invention provide for the exclusion of certain "marker" polypeptides from those proteins that are envisioned as being advantageously coexpressed with the norleucine degrading protein. Thus certain aspects of this

embodiment of the instant invention provide for purification of the coexpressed heterologous protein for advantageous use elsewhere. For example, in one aspect of this embodiment of the invention the heterologous protein is a bovine somatotropin that is to be isolated for use in cattle or another susceptible animal. It is typically important that a heterologous peptide be of its native sequence (or as close thereto as possible) when it is to be used in a higher organism, such as a mammal. For these uses, proteins having minimal norleucine content are most desirable. Similarly, for this reason it is also desirable to express proteins having their native sequence (*i.e.* not mutated to replace methionine with another amino acid, in an effort to prevent norleucine incorporation).

[0058] Proteins that are contemplated as being part of this group of marker peptides include all proteins commonly used by those of ordinary skill in the art as a means of identifying cells that have been transformed. This list includes, but is not limited to antibiotic resistance genes such as ampicillin resistance genes, chloramphenicol acetyl transferase (CAT), tetracycline resistance, kanamycin resistance, neomycin resistance, streptomycin resistance, spectinomycin resistance, gentamicin resistance, and zeocin resistance. This list also includes proteins that are essential for the maintenance of the plasmid, such as proteins involved in plasmid DNA replication, regulation of plasmid copy number, and plasmid mobilization and transfer. This list also includes proteins used to select for the presence of plasmid inserts, such as positive selection markers.

[0059] Various embodiments of the invention provide for the coexpression of any desired heterologous protein in a cell with enhanced expression of one or more norleucine degrading proteins (or norleucine degrading fragments thereof). For example, bST or any other type of ST can be coexpressed in a cell with enhanced expression of wild-type *E. coli* GDH (or with enhanced expression of the K92L *E. coli* GDH variant). Alternatively, a desired heterologous protein can be coexpressed in a cell modified to have enhanced expression of any other norleucine degrading protein or a catalytically active fragment of any norleucine degrading protein.

[0060] Accordingly, one particularly preferred embodiment of the instant invention provides for bST or another somatotropin being coexpressed in *E. coli* with enhanced expression of *E. coli* GDH or enhanced expression of a K92L variant of *E. coli* GDH.

According to various aspects of this embodiment of the invention, the *E. coli* strain may be a K-12 strain or any other strain suitable for protein expression.

[0061] Nevertheless, the methods of the instant invention may be carried out using any desired combination of norleucine degrading protein, heterologous protein, and host cell. That is, the invention is not limited to any particular combinations of cell, norleucine degrading protein, and heterologous protein; rather all possible combinations and/or permutations of the cells, norleucine degrading proteins, and heterologous proteins described herein are envisioned as being part of the instant invention.

[0062] Various embodiments of the instant invention also provide for methods of producing and/or isolating proteins wherein the percent of proteins comprising norleucine has been reduced by at least 50% (as compared with the level of heterologous protein comprising norleucine when the heterologous protein is produced in the same cell type and under the same conditions, except that the cell does not have enhanced expression of a norleucine degrading protein). More preferably, the percent reduction in norleucine content is 60%, 70%, 80%, 90%, or greater than 90%. The percent reduction in norleucine content is typically calculated as a reduction in percentage of proteins containing norleucine. Nevertheless, any suitable method for analyzing the reduction in norleucine content may be used, such as calculating the amount of norleucine present in heterologous proteins isolated from cells that do not have enhanced expression of a norleucine degrading protein and then comparing this result with the amount of norleucine in heterologous proteins present in heterologous proteins isolated from cells grown under identical conditions, except that the cells have enhanced expression of a norleucine degrading protein.

[0063] Other embodiments of the instant invention provide for methods of producing cells that have enhanced expression of a norleucine degrading protein wherein the cells have a decreased pool of norleucine, as compared with the same cells that do not express the norleucine degrading protein, when grown under conditions that are suitable to elicit norleucine production. In preferred aspects of this embodiment of the invention, the amount of norleucine present in the cells' amino acid pool is decreased by at least 20%. In more preferred aspects of this embodiment the amount of norleucine present in the

amino acid pools of the cells is decreased by 30%, 40%, 50%, 60%, 70%, 80%, 90%, or greater than 90%.

[0064] Another method of measuring the reduction in amount of norleucine present is as a function of an increased ratio of methionine to norleucine. In various aspects of this embodiment of the invention the methionine to norleucine ratio is preferably increased to at least 1.2:1, more preferably the ratio is increased to 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, or 1.9:1. More preferably the methionine to norleucine ratio is at least 2.0:1. Even more preferably, the methionine to norleucine ratio is greater than 2.0:1.

[0065] The cell may be of any type suitable for expression of a heterologous protein with simultaneously enhanced expression of a norleucine degrading protein. In a preferred aspect of this embodiment the cell is from an organism that synthesizes norleucine and incorporates it into heterologous protein. In a more preferred embodiment of this aspect of the invention, the cell expresses the norleucine degrading protein at a higher rate than the norleucine degrading protein is expressed in the native cell. In an even more preferred embodiment the cell is an *E. coli* cell.

[0066] In various aspects of the embodiments described above the reduction in the content of the norleucine in heterologous proteins or reduction in norleucine content in the amino acid pool of the cell is accomplished by the enhanced expression of a norleucine degrading protein in the cell, in accordance with the methods described herein. Such enhanced expression may be from a extra-genomic vector such as a plasmid or it may be from a genomic sequence that is not native to the cell, or it may result from a modification of the norleucine degrading protein's native regulatory control mechanism.

[0067] As described, the present invention envisions that these aspects of the invention may be used in any combination with any of the other embodiments described herein. Accordingly, the aspects of this embodiment of the invention include the coexpression of any heterologous protein with any suitable norleucine degrading protein in any suitable cell type. Nevertheless, by way of non-exclusive example, it is noted that preferred embodiments of the invention are drawn to the coexpression of heterologous proteins in a cell with enhanced expression of a norleucine degrading protein selected from one or more of the following: a glutamate dehydrogenase, a phenylalanine dehydrogenase, a

valine dehydrogenase, and a leucine dehydrogenase, other amino acid dehydrogenases, such as alanine dehydrogenase and glycine dehydrogenase, aminotransferases (also known as transaminases), amino acid dehydratases, and various amino acid oxidases. Also contemplated by the instant invention is the use of catalytically active fragments or catalytically active variants of any of the foregoing. In a particularly preferred embodiment of this aspect of the invention the norleucine degrading protein is a glutamate dehydrogenase. In an even more preferred aspect the norleucine degrading protein is *E. coli* glutamate dehydrogenase or a lysine 92 leucine variant of *E. coli* glutamate dehydrogenase. In an even more preferred aspect of this embodiment the glutamate dehydrogenase comprises the amino acid sequence of SEQ IN NO:2 or SEQ ID NO:4. Most preferably, the glutamate dehydrogenase is encoded by a DNA sequence comprising the sequence of SEQ ID NO:1 or SEQ ID NO:3.

[0068] As indicated herein, various embodiments of the instant invention provide heterologous proteins and norleucine degrading protein/protein fragments that are expressed from vectors transformed into a host cell (such as *E. coli*). In certain aspects of this embodiment, the heterologous protein and norleucine degrading protein are expressed from separate plasmids/vectors. In other embodiments they may be expressed from separate portions of the same plasmid or vector. Alternatively, one or both of the heterologous protein and norleucine degrading protein may be expressed from a site that is integral with the host cell's genome.

[0069] In any of the embodiments of the instant invention the expression of the heterologous protein and the enhanced expression of the norleucine degrading protein may be expressed from either constitutive or from inducible promoters. Many constitutive and inducible promoters are well characterized and known to those skilled in the art.

[0070] According to various embodiments of the instant invention the methods are effective to reduce the percentage of heterologous protein containing norleucine to below 5%. In more preferred aspects of this embodiment the percentage of heterologous proteins containing norleucine is decreased to 4%, 3%, 2%, 1%, 0.5%, 0.25%, 0.01%, 0.05% and 0%.

[0071] Although it is not required, the present invention also provides for the simultaneous expression of a heterologous protein with two or more norleucine degrading proteins each of which has enhanced expression. For example, bST can be simultaneously expressed with both wild-type and K92L variant *E. coli* GDH, if desired.

[0072] The instant invention also provides for a recombinant *E. coli* glutamate dehydrogenase protein wherein amino acid residue 92 has been changed from the native lysine to a leucine. In a particularly preferred embodiment the recombinant GDH protein comprises the sequence of SEQ ID NO:4. In an even more preferred embodiment, the GDH protein consists of or consists essentially of the sequence of SEQ ID NO:4.

[0073] Furthermore, if desired the instant invention provides for cells comprising the recombinant *E. coli* GDH comprising the K92L variant. In a preferred aspect of this embodiment the cells are *E. coli* cells. In an even more preferred embodiment, the cells are *E. coli* K-12 cells. Nevertheless, the instant invention is drawn to any cell containing the variant K92L GDH protein, such that it has an enhanced capacity to degrade norleucine.

[0074] The invention also provides for a recombinant DNA capable of encoding the K92L variant of the *E. coli* GDH protein (or an active fraction thereof). A preferred aspect of this embodiment provides for a recombinant DNA molecule comprising the sequence provided as SEQ ID NO:3. Nevertheless one of skill in the art will appreciate that, owing to the degenerate nature of the genetic code, the recombinant DNA sequence may be varied without changing the sequence of the protein encoded thereby. Accordingly, various aspects of this embodiment of the instant invention are drawn to any sequence capable of encoding an *E. coli* K92L GDH variant. Other aspects of this embodiment provide for recombinant DNA sequences encoding *E. coli* K92L GDH variants that further comprise variations at other amino acid residues. These variations are contemplated as being part of the instant invention so long as they do not reduce the ability of the encoded protein to degrade norleucine to a degree that makes it unsuitable for use to prevent or substantially eliminate norleucine incorporation into a heterologous protein expressed in a cell.

[0075] Other embodiments of the instant invention provide for a cell comprising any of the recombinant DNA molecules described herein. In a preferred aspect of this embodiment the cell is an *E. coli* cell. In an even more preferred embodiment the cell is an *E. coli* K-12 cell. Other embodiments provide for cells comprising any of the recombinant DNA molecules described herein wherein enhanced expression of a norleucine degrading protein prevents or substantially eliminates incorporation of norleucine into a heterologous protein coexpressed in the cell.

[0076] Other embodiments of the instant invention provide for methods of producing a protein in and/or isolating a protein from a cell or microorganism. The various embodiments of these methods comprise the use of any combination of the cells, heterologous proteins, and norleucine degrading proteins described herein. The various aspects of this embodiment comprise coexpressing a heterologous protein and in a cell or microorganism with enhanced expression of a norleucine degrading protein and then isolating protein from the microorganism. Preferably, the heterologous protein is isolated from the cell or microorganism. Methods for protein isolation are well known in the art and may be accomplished by means compatible with the selected heterologous protein.

[0077] Other aspects of this embodiment of the invention provide for methods comprising isolating proteins from a cell or microorganism that coexpresses a norleucine degrading protein and a heterologous protein.

## EXAMPLES

[0078] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the Applicant to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed, while still obtaining a like or similar results, without departing from the scope of the invention.

**Example 1: Inhibition of Norleucine incorporation by cotransformation with GDH**

[0079] The wild-type *E. coli* GDH gene was cloned. A K92L variant of this GDH gene was also prepared. Both the wild-type and K92L variant GDH encoding genes were separately cloned into a bovine somatotropin (bST) expression vector (the bST expression vector was designated pXT757) to generate two new plasmids designated pXT814 (comprising wild-type GDH) and pXT815 (comprising K92L variant GDH). In pXT814 the wild-type GDH was placed downstream of the bST gene and in pXT815 the K92L variant was placed downstream of the bST gene. In each of the new plasmids the GDH gene was placed under the control of a constitutive lacUV5 promoter. This was done so that the GDH protein would be constitutively expressed (*i.e.* both before and after induction of bST synthesis (in each plasmid, bST was expressed from an inducible promoter such as the cpex-20 promoter disclosed in WO 00/060103 and WO 02/051238).

[0080] The pXT757, pXT814 and pXT815 plasmids were then separately transformed into the *E. coli* K-12 host strain LBB427 (LBB427 is a derivative of the common K-12 strain, W3110, which has a *fhuA* gene knockout mutation). The transformed strains were grown on minimal medium (*i.e.* no supplemental isoleucine, leucine, methionine, ALIMET®, rich medium supplement, or any other amino acid was added). Next, bST synthesis was induced and the bST protein was isolated and analyzed for norleucine content. The strain transformed with pXT757 was used as a control. The resulting percentages of bST containing norleucine were as shown in Table 1.

**Table 1**

| Host Strain (Plasmid) | Description | Percent of protein containing norleucine |
|---|---|---|
| LBB427 (pXT757) | Control, no coexpressed norleucine degrading protein | 6.1 |
| LBB427 (pXT814) | Coexpressed with Wild-type GDH | 0.9 |
| LBB427 (pXT815) | Coexpressed with K92L variant GDH | 0.6 |

[0081] As the data presented in Table 1 demonstrate, the cloned GDH gene product degrades much of the norleucine, thereby reducing the incorporation of norleucine from 6.1% to 0.9%. Moreover, The K92L variant gene product more effectively reduces the percentage of proteins containing norleucine, to a level of 0.6%.

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[0082] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.


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**WHAT IS CLAIMED IS:**

1. A method for reducing norleucine incorporation in a heterologous protein expressed by microorganisms comprising:
coexpressing in the microorganism at least one heterologous protein; wherein the microorganism has enhanced expression of at least one norleucine degrading protein.

2. The method of claim 1 wherein the norleucine degrading protein is a glutamate dehydrogenase (GDH).

3. The method of claim 2 wherein the glutamate dehydrogenase is from *Escherichia coli*.

4. The method of claim 1 wherein the norleucine degrading protein is a lysine 92 leucine variant of glutamate dehydrogenase.

5. The method of claim 4 wherein the variant glutamate dehydrogenase is from *Escherichia coli*.

6. The method of claim 1 wherein the microorganism is *Escherichia coli*.

7. The method of claim 1 wherein at least one of the expressed heterologous protein(s) is a somatotropin.

8. The method of claim 7 wherein the somatotropin is selected from the group consisting of human, equine, bovine, ovine, porcine, canine, or feline somatotropin.

9. The method of claim 7 wherein the somatotropin is bovine somatotropin.

10. The method of claim 1 wherein the microorganism is *Escherichia coli* (*E. coli*); wherein the norleucine degrading enzyme is *E. coli* glutamate dehydrogenase or a lysine 92 leucine variant of *E. coli* glutamate dehydrogenase; and wherein the heterologous protein is bovine somatotropin.

11. The method of claim 10 wherein the norleucine degrading enzyme is a lysine 92 leucine variant of *Escherichia coli* GDH.

12. The method of claim 1 wherein the heterologous protein and the norleucine degrading protein are expressed from a single expression vector.

13. The method of claim 1 wherein the heterologous protein and the norleucine degrading protein are expressed from at least two distinct expression vectors.

14. A recombinant *Escherichia coli* glutamate dehydrogenase protein comprising a lysine 92 to leucine variation.

15. The recombinant protein of claim 14 having the sequence of SEQ ID NO:4.

16. A recombinant DNA molecule capable of encoding an *Escherichia coli* glutamate dehydrogenase variant; wherein the variant comprises a variation in the codon encoding amino acid 92, as compared to the wild-type DNA sequence, such that it encodes leucine instead of lysine.

17. The DNA molecule of claim 16 comprising the sequence of SEQ ID NO:3.

18. The DNA molecule of claim 16 capable of encoding a protein comprising the sequence of SEQ ID NO:4.

19. An *Escherichia coli* (*E. coli*) cell comprising a DNA sequence encoding an *E. coli* glutamate dehydrogenase; wherein said glutamate dehydrogenase comprises a lysine 92 to leucine variation.

20. The cell of claim 19 wherein the cell comprises a DNA molecule comprising the sequence of SEQ ID NO:3.

21. The cell of claim 19 wherein the cell expresses a protein comprising the sequence of SEQ ID NO:4.

22. A transformed *Escherichia coli* (*E. coli*) cell capable of expressing a norleucine degrading enzyme at a level higher than the enzyme is expressed in a non-transformed precursor cell from which the transformed cell was made.

23. The cell of claim 22 comprising a DNA plasmid for expressing an enzyme capable of degrading norleucine degradation.

24. The cell of claim 22 having a DNA sequence encoding a norleucine degrading enzyme inserted into a non-native position of its genome.

25. The cell of any of claims 22, 23, or 24 wherein the norleucine degrading enzyme is a glutamate dehydrogenase.

26. The cell of claim 25 wherein the glutamate dehydrogenase is an *E. coli* glutamate dehydrogenase.

27. The cell of claim 25 wherein the glutamate dehydrogenase has the sequence of SEQ ID NO:2.

28. The cell of claim 25 wherein the glutamate dehydrogenase is encoded by the DNA sequence of SEQ ID NO:1.

29. A method of isolating a protein from a microorganism comprising:
    a) expressing in a microorganism at least one heterologous protein; wherein the microorganism has enhanced expression for at least one norleucine degrading protein;
    b) isolating the heterologous protein from the microorganism.

30. A method of isolating a protein comprising:
    isolating at least one heterologous protein from a microorganism; wherein said microorganism expresses at least one heterologous protein; wherein the microorganism has enhanced expression for at least one norleucine degrading enzyme.

31. The method of either claim 29 or claim 30 wherein the norleucine degrading enzyme is a glutamate dehydrogenase.

32. The method of claim 31 wherein the glutamate dehydrogenase is an *Escherichia coli* glutamate dehydrogenase.

33. The method of claim 32 wherein the glutamate dehydrogenase comprises a lysine 92 to leucine variation.

34. The method of either claim 29 or claim 30 wherein the microorganism is *Escherichia coli.*

35. The method of claim 34 wherein the norleucine degrading enzyme is a glutamate dehydrogenase.

36. The method of either claim 29 or claim 30 wherein the microorganism is *Escherichia coli* (*E. coli*), wherein the norleucine degrading enzyme is a lysine 92 to leucine variant of *E. coli* glutamate dehydrogenase, and where at least one heterologous protein is a bovine somatotropin.

37. The method of claim 1 wherein the heterologous protein and/or the norleucine degrading protein is expressed from a location in the microorganism's genome.

## ABSTRACT

[0083] The instant invention is drawn to the methods and compositions necessary to provide recombinant proteins with a substantially reduced norleucine content. Various embodiments of the invention provide for the substantial elimination of norleucine from incorporation into recombinant proteins by the enhanced expression of a protein (or the enzymatically active portion of protein) capable of degrading norleucine. In one particular embodiment of the invention, the norleucine is degraded by a glutamate dehydrogenase. Also provided are the cells and DNA constructs for carrying out these methods.

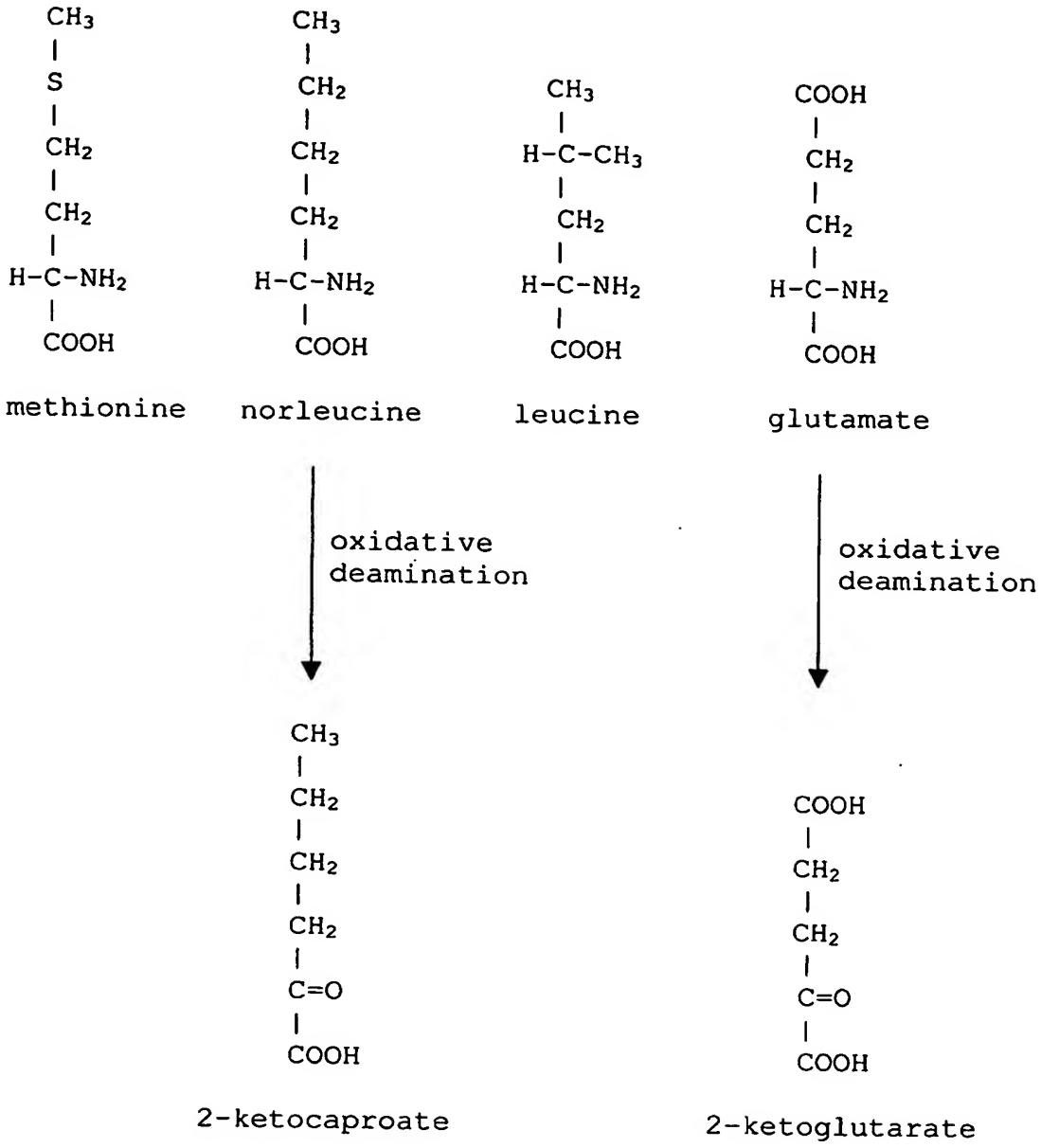
methionine
norleucine
leucine
glutamate
oxidative deamination
oxidative deamination
2-ketocaproate
2-ketoglutarate

**Figure 1**

## SEQUENCE LISTING

Below is the nucleotide sequence of the *E. coli* GDH gene (SEQ ID NO:1); below the nucleotide sequence is the encoded amino acid sequence (SEQ ID NO:2). Lysine residue #92 is indicated in **bold**. In the K92L variant, the lysine codon, AAA, is changed to the leucine codon CTG (the DNA sequence of the variant clone is designated SEQ ID NO:3 and the protein sequence of the K92L variant is SEQ ID NO:4).

```
   1 ATGGATCAGACATATTCTCTGGAGTCATTCCTCAACCATGTCCAAAAGCGCGACCCGAAT  60
     ---------+---------+---------+---------+---------+---------+
     MetAspGlnThrTyrSerLeuGluSerPheLeuAsnHisValGlnLysArgAspProAsn   -

  61 CAAACCGAGTTCGCGCAAGCCGTTCGTGAAGTAATGACCACACTCTGGCCTTTTCTTGAA 120
     ---------+---------+---------+---------+---------+---------+
     GlnThrGluPheAlaGlnAlaValArgGluValMetThrThrLeuTrpProPheLeuGlu   -

 121 CAAAATCCAAAATATCGCCAGATGTCATTACTGGAGCGTCTGGTTGAACCGGAGCGCGTG 180
     ---------+---------+---------+---------+---------+---------+
     GlnAsnProLysTyrArgGlnMetSerLeuLeuGluArgLeuValGluProGluArgVal   -

 181 ATCCAGTTTCGCGTGGTATGGGTTGATGATCGCAACCAGATACAGGTCAACCGTGCATGG 240
     ---------+---------+---------+---------+---------+---------+
     IleGlnPheArgValValTrpValAspAspArgAsnGlnIleGlnValAsnArgAlaTrp   -

 241 CGTGTGCAGTTCAGCTCTGCCATCGGCCCGTAC AAA GGCGGTATGCGCTTCCATCCGTCA 300
     ---------+---------+---------+---------+---------+---------+
     ArgValGlnPheSerSerAlaIleGlyProTyr Lys GlyGlyMetArgPheHisProSer   -
                                       92

 301 GTTAACCTTTCCATTCTCAAATTCCTCGGCTTTGAACAAACCTTCAAAAATGCCCTGACT 360
     ---------+---------+---------+---------+---------+---------+
     ValAsnLeuSerIleLeuLysPheLeuGlyPheGluGlnThrPheLysAsnAlaLeuThr   -

 361 ACTCTGCCGATGGGCGGTGGTAAAGGCGGCAGCGATTTCGATCCGAAAGGAAAAAGCGAA 420
     ---------+---------+---------+---------+---------+---------+
     ThrLeuProMetGlyGlyGlyLysGlyGlySerAspPheAspProLysGlyLysSerGlu   -

 421 GGTGAAGTGATGCGTTTTTGCCAGGCGCTGATGACTGAACTGTATCGCCACCTGGGCGCG 480
     ---------+---------+---------+---------+---------+---------+
     GlyGluValMetArgPheCysGlnAlaLeuMetThrGluLeuTyrArgHisLeuGlyAla   -

 481 GATACCGACGTTCCGGCAGGTGATATCGGGGTTGGTGGTCGTGAAGTCGGCTTTATGGCG 540
     ---------+---------+---------+---------+---------+---------+
     AspThrAspValProAlaGlyAspIleGlyValGlyGlyArgGluValGlyPheMetAla   -

 541 GGGATGATGAAAAAGCTCTCCAACAATACCGCCTGCGTCTTCACCGGTAAGGGCCTTTCA 600
     ---------+---------+---------+---------+---------+---------+
     GlyMetMetLysLysLeuSerAsnAsnThrAlaCysValPheThrGlyLysGlyLeuSer   -
```

```
601 TTTGGCGGCAGTCTTATTCGCCCGGAAGCTACCGGCTACGGTCTGGTTTATTTCACAGAA 660
    ---------+---------+---------+---------+---------+---------+
    PheGlyGlySerLeuIleArgProGluAlaThrGlyTyrGlyLeuValTyrPheThrGlu   -

661 GCAATGCTAAAACGCCACGGTATGGGTTTTGAAGGGATGCGCGTTTCCGTTTCTGGCTCC 720
    ---------+---------+---------+---------+---------+---------+
    AlaMetLeuLysArgHisGlyMetGlyPheGluGlyMetArgValSerValSerGlySer   -

721 GGCAACGTCGCCCAGTACGCTATCGAAAAAGCGATGGAATTTGGTGCTCGTGTGATCACT 780
    ---------+---------+---------+---------+---------+---------+
    GlyAsnValAlaGlnTyrAlaIleGluLysAlaMetGluPheGlyAlaArgValIleThr   -

781 GCGTCAGACTCCAGCGGCACTGTAGTTGATGAAAGCGGATTCACGAAAGAGAAACTGGCA 840
    ---------+---------+---------+---------+---------+---------+
    AlaSerAspSerSerGlyThrValValAspGluSerGlyPheThrLysGluLysLeuAla   -

841 CGTCTTATCGAAATCAAAGCCAGCCGCGATGGTCGAGTGGCAGATTACGCCAAAGAATTT 900
    ---------+---------+---------+---------+---------+---------+
    ArgLeuIleGluIleLysAlaSerArgAspGlyArgValAlaAspTyrAlaLysGluPhe   -

901 GGTCTGGTCTATCTCGAAGGCCAACAGCCGTGGTCTCTACCGGTTGATATCGCCCTGCCT 960
    ---------+---------+---------+---------+---------+---------+
    GlyLeuValTyrLeuGluGlyGlnGlnProTrpSerLeuProValAspIleAlaLeuPro   -

961 TGCGCCACCCAGAATGAACTGGATGTTGACGCCGCGCATCAGCTTATCGCTAATGGCGTT 1020
    ---------+---------+---------+---------+---------+---------+
    CysAlaThrGlnAsnGluLeuAspValAspAlaAlaHisGlnLeuIleAlaAsnGlyVal   -

1021 AAAGCCGTCGCCGAAGGGGCAAATATGCCGACCACCATCGAAGCGACTGAACTGTTCCAG 1080
     ---------+---------+---------+---------+---------+---------+
     LysAlaValAlaGluGlyAlaAsnMetProThrThrIleGluAlaThrGluLeuPheGln   -

1081 CAGGCAGGCGTACTATTTGCACCGGGTAAAGCGGCTAATGCTGGTGGCGTCGCTACATCG 1140
     ---------+---------+---------+---------+---------+---------+
     GlnAlaGlyValLeuPheAlaProGlyLysAlaAlaAsnAlaGlyGlyValAlaThrSer   -

1141 GGCCTGGAAATGGCACAAAACGCTGCGCGCCTGGGCTGGAAAGCCGAGAAAGTTGACGCA 1200
     ---------+---------+---------+---------+---------+---------+
     GlyLeuGluMetAlaGlnAsnAlaAlaArgLeuGlyTrpLysAlaGluLysValAspAla   -

1201 CGTTTGCATCACATCATGCTGGATATCCACCATGCCTGTGTTGAGCATGGTGGTGAAGGT 1260
     ---------+---------+---------+---------+---------+---------+
     ArgLeuHisHisIleMetLeuAspIleHisHisAlaCysValGluHisGlyGlyGluGly   -

1261 GAGCAAACCAACTACGTGCAGGGCGCGAACATTGCCGGTTTTGTGAAGGTTGCCGATGCG 1320
     ---------+---------+---------+---------+---------+---------+
     GluGlnThrAsnTyrValGlnGlyAlaAsnIleAlaGlyPheValLysValAlaAspAla   -

1321 ATGCTGGCGCAGGGTGTGATTTAA 1344
     ---------+---------+----
     MetLeuAlaGlnGlyValIleEnd
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